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Review

Detection of Disseminated Melanoma Cells by Reverse-Transcription – Polymerase Chain Reaction

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A B S T R A C T

Detection of circulating melanoma cells by reverse transcriptase – polymerase chain reaction (RT-PCR) is a molecular diagnostic procedure which is used to predict prognosis in melanoma patients. The most widely used specific marker for detection of circulating melanoma cells by RT-PCR is expression of tyrosinase gene. This procedure has shown high specificity and low threshold for detection of melanoma cells. Most of the studies have shown that prognosis is worse in patients in which circulating melanoma cells were detected. Detection of circulating melanoma cells has been studied also as a marker for predicting response to therapy. The clinical value of this procedure is limited by the proportion of patients with clinically confirmed distant metastases being tyrosinase negative in almost all the studies. Studies have shown that analysis of additional markers to tyrosinase enables detection of circulating melanoma cells in a higher percentage of melanoma patients. RT-PCR has shown a lower threshold for detection than other methods (immunohistochemistry) in detection of melanoma metastases in lymph nodes.

***Key words**: melanoma, neoplasm circulating cells, neoplasm metastasis, reverse transcriptase polymerase chain reaction, tumor markers – biological*

Introduction

There are two principal reasons for melanoma being a major healthcare problem nowadays. One is the significant increase in melanoma incidence worldwide in last decades[1,2]. The other is that, in spite of intense research, no treatment has confirmed significant influence on survival of advanced stage melanoma patients. The extent of the healthcare problem implies the need for new, more accurate and reliable diagnostic procedures that would help in diagnosis, predicting prognosis and the follow-up of melanoma patients. The detection of circulating melanoma cells is one such molecular-diagnostic procedure, which is based on analysis of tumor markers.

Tumor Markers for Melanoma Diagnosis

Tumor markers can be defined as compounds the presence or change in concentration of which are associated with the presence or the progression of tumors. Tumor markers have different applications. They can be used for screening of healthy or high-risk populations to predict development of tumors[3]. For that purpose the most widely investigated marker for melanoma are mutations in cycline-dependent kinase inhibitor 2A (CDKN2A) gene, but still the routine analysis of this marker is not recommended[4,5].

Tumor markers can be used for making diagnosis of specific type of cancer[3]. For that purpose the immunohistochemical analysis of tumor markers is most widely applied. Immunohistochemical analysis is applied in cases in which the standard histopathological analysis (staining of specimens with hematoxylin and eosin) can not reliably confirm the diagnosis of melanoma. Immunohistochemical analysis is also applied for detection of melanoma metastases in regional lymph nodes, which is

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of critical importance for predicting the course of the disease and deciding on therapy. For immunohistochemical analysis of melanoma the most widely used markers are HMB45 antibodies specific for gp100 protein and antibodies specific for S100 protein[6].

Finally, tumor markers can be used for determining prognosis and following the course of the disease in patients who are in remission (for early detection of metastases and relapse) or are receiving therapy (to follow the response to therapy)[3]. For that purpose the serum tumor markers and detection of circulating tumor cells are most often used. The most widely applied serum melanoma markers are enzyme lactate dehydrogenase (LDH), S100-β, and melanoma inhibitory activity (MIA)[7–9]. Increased serum concentration of LDH is included in the American Joint Committee on Cancer (AJCC) staging instructions from 2002 as an independent prognostic factor[10]. Many other molecules are being investigated as potential prognostic markers in patients with melanoma[7,9] (Table 1).

The development of new cytogenetic methods (comparative genomic hybridization (CGH), and fluorescent *in situ* hybridization (FISH)), molecular genetic methods (e.g. loss of heterozygosity (LOH) analysis), and genomics, transcriptomics and proteomics has enabled new approaches to the analysis of tumor markers in patients with melanoma[11,12]. All these methods in addition to providing new insights into processes of cancer development and its biology also offer the possibility of analysis of changes on the DNA and RNA level and on the whole genome, transcriptome and proteome level which can provide us with information useful for establishing diagnosis and determining prognosis in patients with melanoma[11,12].

Detection of Circulating Melanoma Cells – Method, Specificity and Threshold for Detection

On the basis of the assumption that circulating melanoma cells are associated with the development of distant metastases, the presence of circulating melanoma cells is investigated as a potential prognostic marker in patients with melanoma. Nevertheless, the real biological and clinical significance of circulating tumor cells has not been completely revealed. It has been shown that less then 0.1% of circulating melanoma cells develop distant metastases[13].

Before reverse-transcription – polymerase chain reaction (RT-PCR) was introduced, researchers had tried to detect circulating tumor cells using cytological and immunocytochemical methods[14]. But the threshold for detection of these methods was not low enough. Only the introduction of RT-PCR enabled reaching the threshold for detection low enough to detect small number of circulating tumor cells[15]. Smith et al were first to show in 1991 that circulating melanoma cells can be detected by RT-PCR using tyrosinase mRNA as a specific marker[15].

Tyrosinase (monophenol monooxygenase, EC 1.14. 18.1) is an enzyme that catalyses two important steps in melanin biosynthesis, oxidation of 3,4-dihidrohyphenylalanine (DOPA) to dopaquinone and synthesis of dopachrome[16] (Figure 1). Tyrosinase is specifically expressed by melanocytes, Schwann cells and melanoma cells[17,18]. Since melanocytes and Schwann cells are not normally present in the circulation, the detection of tyrosinase mRNA in the peripheral blood of patient with melanoma indicates the presence of melanoma cells.

Numerous studies that followed after publication of results by Smith et al, confirmed a low threshold for detection and high specificity of this method. In the majority of studies the threshold for detection was low enough to allow for the detection of 1 to 10 melanoma cells in 10 mL of peripheral blood in the relative ratio of 1 to 10 melanoma cells in 10^7 peripheral blood mononuclear cells[15,19–37]. It has been shown that the threshold for detection of circulating melanoma cells depends significantly on the method used for blood sample processing. Schittek et al compared four different blood sample processing methods which had been used in previous studies of circulating melanoma cell detection by RT-PCR[20]. Using direct

TABLE 1
MOLECULES INVESTIGATED AS PROGNOSTIC IMMUNOHISTOCHEMICAL AND SERUM MARKERS IN PATIENTS WITH MELANOMA

Markers of cellular proliferation	Ki-67*
Tumor suppressor genes	p53*, p16*, p21*, p27*
Oncogenes	c-myc*
Adhesion molecules	integrins*, CD44*, MUC-18*, E-cadherin*
Molecules involved in enzymatic extracellular matrix degradation	metalloproteinases* (MMP-1, 2, 9 and 13) tissues inhibitors of metalloproteinases* (TIMP-1, 2 and 3) plasminogen activators* (uPA, tPA)
Melanoma antigenes	neuron-specific enolase**, lipid-associated sialic acid**, microphthalmia-associated transcription factor*
Melanin metabolites	5-S-cysteinyldopa**, 6-hydroxy-5-methoxyindole-2-carboxylic acid**
Cytokines and cytokine receptors	IL-6**, IL-8**, IL-10**, soluble IL-2 receptor**

* immunohistochemical markers, ** serum markers

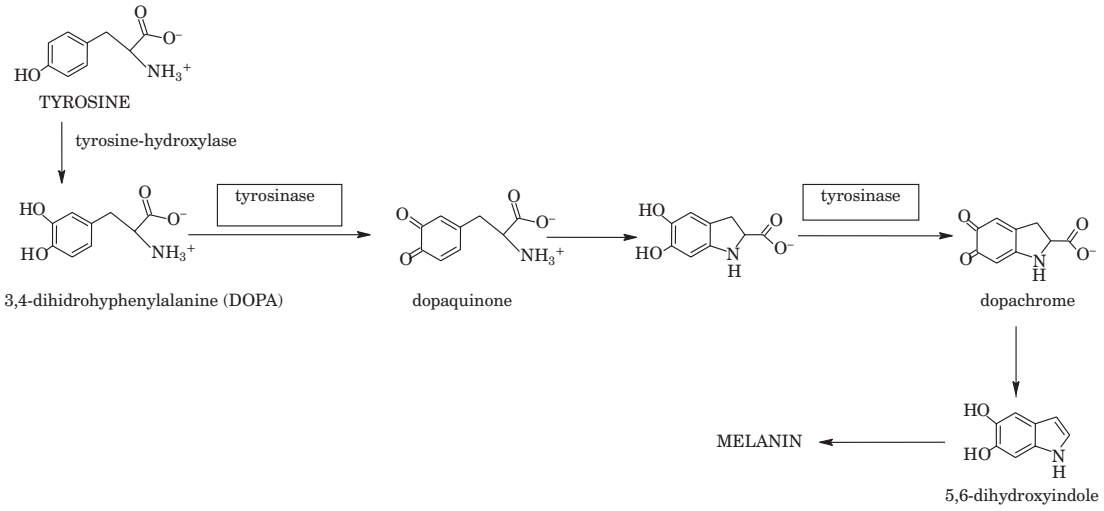

Fig. 1. Role for tyrosinase in melanin biosynthesis.

isolation of RNA from total blood they could detect a minimum of 300 melanoma cells added to a blood sample. When erythrocytes were lysed prior to RNA isolation they could detect a minimum of 50 melanoma cells added to a blood sample, but only in one of five blood samples analyzed. The lowest threshold for detection was achieved when RNA was isolated from mononuclear cells that had been isolated from blood sample using a ficoll gradient with a density of 1.077 g/mL. Using this method a minimum of two melanoma cells was detected in 60% of blood samples analyzed and a minimum of 40 melanoma cells was detected in all blood samples analyzed. Changing the density of the ficoll gradient to 1.09 g/mL resulted in a higher threshold for detection in comparison with the density of 1.077 g/mL. Jung et al have also shown that isolation of mononuclear cells in the ficoll gradient prior to RNA isolation results in a lower threshold for melanoma cell detection than direct isolation of RNA from total blood[36].

In most of the studies all blood samples from donors that did not have melanoma were negative for tyrosinase, which shows high specificity of this method[15,19,21–25,27–40]. Only two of 512 negative controls (healthy volunteers and patients with non-melanoma cancer) in 23 different studies analyzed in a meta-analysis by Tsao et al were positive for tyrosinase[35].

Detection of Circulating Melanoma Cells – Clinical Application

In some studies the correlation of detection of circulating melanoma cells with the stage of disease was found (Figure 2). The proportion of patients with positive value of marker (mostly tyrosinase) was higher in patients with higher stage of the disease[21,22,25,32,33,37,39–43] (Figure 2). Still, in some other studies the correlation of detection of circulating melanoma cells with the stage of the disease was not found[19,29,30,34,44,45]. The proportion of patients positive for tyrosinase in each stage of the disease varied significantly between studies. Tsao et al analyzed 23 different studies on 1799 patients and obtained the following average values for percentage of patients positive for tyrosinase: 18% (95% confidence interval, 3–22%) for patients in stage 1, 28% (95 confidence interval, 23–34%) for patients in stage 2, 30% (95% confidence interval, 26–34%) for patients in stage 3, and 45% (95% confidence interval, 41–50%) for patients in stage 4 according to the AJCC[35] (Figure 2).

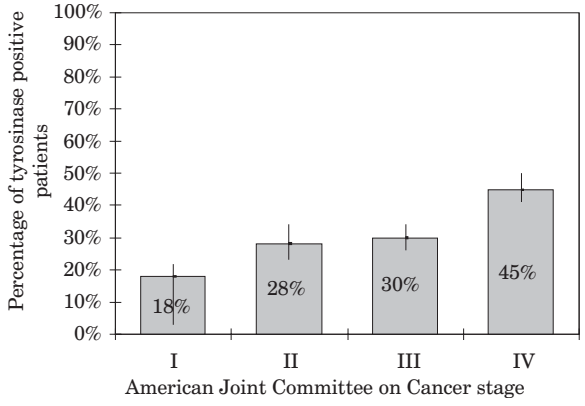

Fig. 2. Percentage of melanoma patients positive for tyrosinase in different American Joint Committee on Cancer stages. Percentages were calculated from data in 23 different studies analyzed in meta-analysis by Tsao et al.[35].

The prognostic value of detection of circulating melanoma cells was also investigated. In most of these studies patients positive for tyrosinase had significantly increased risk for disease recurrence and shorter survival[23,26,28–34,39,40,42–44,46]. Still, some other studies did not confirm the prognostic value of detection of circulating melanoma cells in patients with melanoma[25,27,45].

Some studies performed on patients receiving adjuvant therapy have shown that detection of circulating melanoma cells can be used as a marker to follow the re-

sponse to therapy[29,30,38,41,44,46]. Reynolds et al analyzed four markers (tyrosinase, gp100, MAGE-3 and MelanA/MART1) for detection of circulating melanoma cells in 118 melanoma patients treated adjuvantly with polyvalent melanoma vaccine[44]. Blood samples for analysis were collected before treatment and 3, 5, and 11 months after the initiation of treatment (after the fifth, seventh and ninth immunization, respectively). Results of this study have shown that therapy is associated with the clearance of melanoma cells from the circulation (after three and five months of therapy the proportion of patients with positive value of markers decreased by 27% and 55%, respectively)[44]. The clearance of melanoma cells from circulation during the therapy detected in this study was associated with better prognosis[44]. Schmidt et al have also shown that the proportion of patients with positive value of markers (tyrosinase and MelanA/MART1) is significantly lower during the adjuvant treatment (interleukin-2, interferon-α2b and cisplatin) than before the treatment[38]. In this study loss of expression of markers during the treatment was not associated with better prognosis[38]. Gogas et al have shown that the risk for relapse was significantly lower in patients that were positive for tyrosinase before adjuvant treatment (interferon) and negative during and after the treatment than in patients that were positive for tyrosinase during and after the treatment[30]. Mellado et al have shown that patients with melanoma and tyrosinase mRNA detected in blood during adjuvant interferon therapy had significantly shorter overall and disease-free survival than melanoma patients with undetected tyrosinase mRNA during treatment[29]. The detection of tyrosinase mRNA in blood before interferon treatment was not associated either with overall or with disease-free survival in the same study[29]. Koyanagi et al have shown that the detection of mRNA for microphtalmia-associated transcription factor (MITF) as a marker for circulating melanoma cells after adjuvant biochemotherapy (cisplatin, dacarbazine, vinblastine, interleukin-2, interferon, and granulocyte colony-stimulating factor (G-CSF)) is a significant independent prognostic factor for relapse-free and overall survival[41]. They have also shown that the proportion of patients positive for MITF was significantly lower after the therapy than before the therapy in patients who remained relapse-free during follow-up, but not in patients who had a relapse[41]. In another study Koyanagi et al analyzed four markers for detection of circulating melanoma cells (MelanA/MART1, PAX-3, MAGE-A3, and GalNAc-T) in melanoma patients treated with adjuvant biochemotherapy (cisplatin, dacarbazine, vinblastine, interleukin-2, interferon, and G-CSF)[46]. In this study they have shown that the proportion of patients with positive values of marker is significantly lower after the treatment than before, and that positive value of markers after the treatment (but not before the treatment) is significantly associated with shorter relapse-free and overall survival[46].

TABLE 2
PROPORTION OF MELANOMA PATIENTS WITH DISTANT METASTASES AND POSITIVE FINDING OF TYROSINASE IN PERIPHERAL BLOOD BY REVERSE-TRANSCRIPTION – POLYMERASE CHAIN REACTION IN DIFFERENT STUDIES

Study	Tyrosinase positive patients with distant metastases	
	Number/Total number of patients with distant metastases tested	Percentage
Smith et al. (1991)[15]	4/6	67%
Battayani et al. (1995)[31]	16/32	50%
Mellado et al. (1996)[32]	33/35	94%
Reinhold et al. (1997)[26]	5/13	39%
Jung et al. (1997)[36]	13/50	26%
Curry et al. (1998)[21]	60/116	52%
Schittek et al. (1999)[39]	21/58	36%
Schrader et al. (2000)[23]	13/80	16%
Proebstle i sur. (2000)[33]	16/24	67%
Brownbridge et al. (2001)[25]	30/37	81%
Quereux et al. (2001)[27]	20/32	63%
Mellado et al. (2002)[29]	5/15	33%
Schmidt et al. (2002)[38]	15/35	43%
Reynolds et al. (2003)[44]	3/11	27%
Jin et al. (2003)[37]	5/9	56%
Palmieri et al. (2003)[43]	15/23	65%
Šamija et al. (2004)[24]	5/13	38%
Ranieri et al. (2005)[45]	2/2	100%

Detection of Circulating Melanoma Cells – Negative Value of Markers in Patients with Distant Metastases

The fact that in almost all the studies a proportion of patients with clinically confirmed distant metastases were negative for tyrosinase makes clinical relevance of circulating melanoma cells detection disputable[15,18,21,23–25,26,27,29,32,33,35–40,43,44] (Table 2). Three different mutually non-exclusive explanations of these results have been proposed.

Reinhold et al have shown that among blood samples taken from the same melanoma patient in 2-hours intervals some were positive and some were negative for tyrosinase[26]. In order to explain this result they proposed an explanation for negative finding of tyrosinase in peripheral blood from patients with distant metastases based on the model of the metastatic process according to which tumor cells are shed into the bloodstream transiently, in a random, discontinuous manner[26]. According to this explanation, the clinical value of circulating melanoma cells detection could be improved by analyzing several blood samples taken from the same patient at different time points. However, this is difficult to carry out in practice, because single blood samples are usually taken during routine follow-up visits.

Szenajch et al have shown that the probability of positive finding of tyrosinase in peripheral blood of melanoma patients increases proportionally with the number of blood samples analyzed *per* patient[47]. Based on this finding they speculated that melanoma cells are present in circulation of almost all melanoma patients, but their number is below the threshold for detection of the method[47]. In that case the clinical value of circulating melanoma cells detection could be improved if a larger volume of blood was analyzed (around 50 mL instead of usual 5–10 mL) or if more blood samples were analyzed *per* patient[47]. Both of these are approaches difficult to carry out in practice.

It is possible that a negative finding of tyrosinase in the peripheral blood of melanoma patients with distant metastases is the result of complete loss or a significant decrease in the expression of tyrosinase gene in a proportion of melanoma cells in the course of tumor progression due to dedifferentiation[19,39]. Chen et al have found different levels of tyrosinase expression in different melanoma tissue specimens, a small proportion of them even being negative[48]. Consequentially, the clinical value of detection of circulating melanoma cells could be improved by analysis of additional markers to tyrosinase. Different studies indeed have shown that the proportion of melanoma patients with a positive finding of circulating melanoma cells increased when additional markers to tyrosinase were analyzed. Kulik et al have shown on a group of 80 melanoma patients that additional analysis of MelanA/MART1, in comparison with tyrosinase analysis alone, enables the detection of circulating melanoma cells in 10% more melanoma patients[19]. In a study performed by Schittek et al on 225 melanoma patients, 72 patients (32.0%) were positive for MelanA/MART1 or tyrosinase, while 50 patients (22.2%) were positive for tyrosinase, which means that additional analysis of MelanA/MART1 increased the sensitivity of melanoma cells detection by 30%[39]. Reynolds et al analyzed four different markers (MelanA/MART1, gp100, MAGE3, and tyrosinase) in a group of 118 melanoma patients and have shown that 10–21% (depending on a marker) patients were positive when only one marker was examined, while 47% were positive when all four markers were examined[44]. Other studies have also shown that analysis of other markers in addition to tyrosinase allows for detection of circulating melanoma cells in a larger percentage of melanoma patients than tyrosinase analysis alone[21,23,43]. Hoon et al have shown that analysis of four markers (tyrosinase, p97, MUC18, and MAGE3) is statistically significantly better than analysis of tyrosinase alone[22]. They have also found that the number of positive markers was statistically significantly correlated with the stage of disease and was a significant independent variable for predicting disease recurrence[22,42]. Unlike all these studies, Brownbridge et al have shown in a group of 211 melanoma patients that analysis of MelanA/MART1 in addition to tyrosinase does not allow for detection of circulating melanoma cells in a larger number of melanoma patients than tyrosinase analysis alone[25]. In this study all the patients that were positive for MelanA/MART1 were also positive for tyrosinase[25].

In addition to tyrosinase and MelanA/MART1, MIA, MAGE3, tyrosinase related protein 2 (TRP2), and MITF have shown high specificity and low threshold for detection as markers for detection of circulating melanoma cells by RT-PCR[22–24,38,41,49].

Other molecules (MUC18, p97, and gp100) that were analyzed as potential markers for detection of melanoma cells by RT-PCR were found positive in healthy subjects, which makes them unreliable as melanoma markers[19,21,22,39].

Detection of Melanoma Cells in Lymph Nodes, Bone Marrow and Cerebrospinal Fluid

Metastatic involvement of regional lymph nodes is an important prognostic factor in patients with melanoma. Histopathologically confirmed presence of metastases in regional lymph nodes was found to be the most significant independent prognostic factor in melanoma patients that did not have clinically evident regional lymph node metastases[50].

The usual standard procedure for detection of melanoma metastases in regional lymph nodes involves staining of lymph node sections with hematoxylin and eosin, and inspection for melanoma cells under light microscope (HE staining)[51,52]. This procedure allows for detection of a single tumor cell among 10^4 healthy cells[51] (Table 3). It has been shown that immunohistochemical analysis allows for detection of regional lymph node metastases in 20% more melanoma patients than standard histopathology (HE staining)[51]. The markers most widely used for immunohistochemical analysis are S100 and gp100 (HMB-45 antibodies).

TABLE 3
THRESHOLD FOR DETECTION OF DIFFERENT PROCEDURES FOR DETECTION OF MELANOMA METASTASES IN LYMPH NODES

Procedure	Threshold for detection*
HE staining	10^4
Immunohistochemistry	10^5
RT-PCR	10^6–10^7

* number of normal lymphocytes between which one melanoma cell can be detected, HE – haematoxyline-eosin, RT-PCR – reverse transcription-polymerase chain reaction

Both HE staining and immunohistochemical analysis are based on the examination of a limited number of sections, allowing for analysis of only a small part of the whole lymph node[51]. It is possible that small groups of melanoma cells which are not present in examined sections are missed that way. This is corroborated by the fact that a group of patients in whom no regional lymph node metastases had been found by HE staining and

immunohistochemical analysis still later developed distant and/or regional lymph node metastases[51,53–56]. RT-PCR has been investigated for detection of regional lymph node metastases in melanoma patients with the aim to single out that particular group of patients. RT-PCR allows for detection of a single melanoma cell among 10^6 to 10^7 healthy cells[51] (Table 3). RT-PCR method theoretically also allows for the analysis of the whole lymph node.

RT-PCR allows for the detection of regional lymph node metastases in a significantly higher proportion of melanoma patients than HE staining and immunohistochemical analysis[52–55,57–60]. Patients in whom the metastases have been found only by RT-PCR and not by HE staining or immunohistochemistry had a significantly shorter survival and higher risk of recurrence than patients in whom metastases were not found either by RT-PCR or by HE staining and immunohistochemistry[52,55,59]. These results indicate that RT-PCR allows for detection of clinically relevant metastases that can not be detected by HE staining or immunohistochemical methods.

Nevertheless, the majority of patients in whom regional lymph node metastases had been detected by RT-PCR did not have relapse, a finding which diminishes the clinical significance of this procedure[51–53,55]. One explanation for these results may be that sentinel lymph nodes in which the metastases were discovered by RT-PCR were the only one with metastases, so the sentinel lymph node biopsy was actually a therapeutic procedure that rendered patients free of disease[51]. A proportion of positive findings of melanoma metastases in regional lymph nodes by RT-PCR can be considered false positive[51,60]. In around 5% of lymph nodes benign nevus cells that express tyrosinase gene can be found, and tyrosinase mRNA was analyzed as melanoma marker in most of these studies[51,60]. This is one of the reasons why analysis of multiple melanoma markers (tyrosianse, MelanA/MART1, MAGE3, gp100, and others) for the detection of melanoma metastases in regional lymph nodes by RT-PCR is studied[51,59–62].

Detection of melanoma cells in bone marrow by RT-PCR has not shown higher sensitivity than detection of melanoma cells in peripheral blood and has not shown prognostic significance[63–65].

In one study, analysis of tyrosinase and two other melanoma markers (MAGE3, and MelanA/MART1) was used for the detection of melanoma cells in cerebrospinal fluid[66]. In this study RT-PCR analysis allowed for detection of melanoma cells in a higher proportion of patients than cytological and immunocytochemical analysis[66]. In the same study significant correlation between the detection of melanoma cells in cerebrospinal fluid by RT-PCR and development of central nervous system metastases in the next three months was found[66].

Conclusion

RT-PCR has shown high specificity and low threshold for detection of circulating melanoma cells, but still further studies are needed before this method can be included in routine clinical practice. One critical point is the standardization of the method and markers in order to make results obtained in different institutions fully comparable. Other critical point is how to incorporate this method in the treatment strategy and algorithms for melanoma patients. Although most of the studies have shown prognostic value of this method, it is not clear at which point and in which patients should it be performed. There are several potential clinical applications of this method. Detection of circulating melanoma cells could be included as a parameter in the staging of melanoma patients in order to better predict survival groups. However, further studies that would confirm that detection of circulating melanoma cells is a prognostic factor independent of clinical stage and other prognostic factors are needed to justify such clinical application of this method. Other potential clinical application is for following melanoma patients after surgery. Positive finding of circulating melanoma cells in otherwise disease free patients could indicate higher risk for metastases, and these patients could be followed more frequently and more thoroughly (eg. inclusion of additional radiological procedures). Starting adjuvant therapy based on positive finding of circulating melanoma cells is not supported by the results published so far. Another potential clinical application of this method is for following a response to adjuvant therapy. Theoretically, the dosage and duration of adjuvant therapy could be modified based on the results of circulating melanoma cells detection. However, further studies aimed specifically at each potential clinical application are needed before decision on clinical use of this method can be made.

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ODREĐIVANJE DISEMINIRANIH MELANOMSKIH STANICA METODOM LANČANE REAKCIJE POLIMERAZOM NAKON OBRNUTOG PREPISIVANJA

S A Ž E T A K

Određivanje cirkulirajućih melanomskih stanica metodom lančane reakcije polimerazom nakon obrnutog prepisivanja (RT-PCR) je molekularno dijagnostički postupak koji se primjenjuje u bolesnika sa melanomom u svrhu predviđanja prognoze. Kao specifični biljeg za određivanje cirkulirajućih melanomskih stanica metodom RT-PCR najčešće se primjenjuje ekspresija gena za tirozinazu. Ovaj postupak je pokazao visoku specifičnost i nizak prag detekcije melanomskih stanica. Većina istraživanja je pokazala de je prognoza lošija u bolesnika u kojih su nađene cirkulirajuće melanomske stanice. Određivanje cirkulirajućih melanomskih stanica istražuje se i kao biljeg za praćenje odgovora ne liječenje. Kliničku vrijednost ovog postupka umanjuje to što je u gotovo svim istraživanjima određeni udio bolesnika s klinički potvrđenim udaljenim metastazama bio negativan na tirozinazu. Pokazalo se da analiza dodatnih biljega uz tirozinazu omogućuje otkrivanje cirkulirajućih melanomskih stanica u većeg broja bolesnika sa melanomom. Metoda RT-PCR je pokazala niži prag detekcije od drugih postupaka (imunohistokemije) pri određivanju metastaza melanoma u limfnim čvorovima.